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Survival and Enumeration of the Fecal Indicators *Bifidobacterium adolescentis* and *Escherichia coli* in a Tropical Rain Forest Watershed

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The density of *Bifidobacterium* spp., fecal coliforms, *Escherichia coli*, and total anaerobic bacteria, acridine orange direct counts, percentages of total bacterial community activity and respiration, and 12 physical and chemical parameters were measured simultaneously at six sites for 12 months in the Mameyes River rain forest watershed, Puerto Rico. The densities of all bacteria were higher than those reported for uncontaminated temperate rivers, even though other water quality parameters would indicate that all uncontaminated sites were oligotrophic. The highest densities for all indicator bacteria were at the site receiving sewage effluent; however, the highest elevation site in the watershed had the next highest densities. Correlations between bacterial densities, nitrates, temperature, phosphates, and total phosphorus indicated that all viable counts were related to nutrient levels, regardless of the site sampled. In situ diffusion chamber studies at two different sites indicated that *E. coli* could survive, remain physiologically active, and regrow at rates that were dependent on nutrient levels of the ambient waters. *Bifidobacterium adolescentis* did not survive at either site but did show different rates of decline and physiological activity at the two sites. Bifidobacteria show promise as a better indicator of recent fecal contamination in tropical freshwaters than *E. coli* or fecal coliforms; however, the YN-6 medium did not prove to be effective for enumeration of bifidobacteria. The coliform maximum contaminant levels for assessing water usability for drinking and recreation appear to be unworkable in tropical freshwaters.

Since 1914, coliforms have been used as the standard indicator of recent fecal contamination in the United States (1). The coliform has proved to be an acceptable indicator under most conditions in temperate freshwater. However, more and more, the "maximum contaminant levels"(MCL) established for temperate areas have been accepted without question by tropical nations. Despite this trend, there is a growing body of evidence that the underlying assumptions of the assays being used are not valid in tropical climates (4).

The waters of Puerto Rico are believed to be grossly contaminated by human waste. The U.S. Water Resources Council reported that 96% of all sampling stations on 26 rivers in Puerto Rico demonstrated violations of the coliform standards (31). As proof of the contamination problem, they cite 7,800 cases of acute gastroenteritis that occurred the previous year, 1977, in Comerio, Puerto Rico due to improper chlorination of drinking water. In fact, few epidemics like this have occurred on the island in the last 10 years, despite the contamination indicated by the coliform assays. Other studies in tropical freshwater have shown that a high proportion of fecal coliform-positive isolates are of nonfecal origin (4, 20). In Puerto Rico, less than 30% of the fecal coliform-positive isolates from a variety of sites around the island are identified as *Escherichia coli* (14, 16; T. C. Hazen, unpublished data). One such site is the Luquillo Experimental Forest, in which the upper parts of some of the forest watersheds are known to have high fecal coliform counts in the absence of any identifiable fecal source (14).

Recent studies have suggested that bifidobacteria may be an excellent candidate as an alternative indicator of fecal contamination (9, 28). It is found in the gut of all humans within the first 6 days of neonatal life (24, 27). It also is one of the dominant anaerobes in the gut of humans, typically reaching densities greater than 10^{10} cells per g of feces. Since bifidobacteria are also obligate anaerobes, they are incapable of surviving in the oxygenated extraenteral environment. A recently developed medium was reported to allow rapid and accurate enumeration of bifidobacteria from environmental samples (28). Thus, it would seem that bifidobacteria would fit all criteria for a good indicator bacterium as described by Bonde (4). The present study examines the distribution, density, and in situ survival of *E. coli* and *Bifidobacterium* spp. in a tropical rain forest watershed in Puerto Rico.

MATERIALS AND METHODS

Study site. The Mameyes river watershed is on the northeast corner of the island of Puerto Rico, lat. 18° 15′ N and long. 65° 45′ W (Fig. 1). This watershed has a drainage area of 27.3 km^2 and a total length of 17.1 km (14). Annual average precipitation in the upper third of the watershed is 395 cm. This area is classified as a cloud rain forest and is protected as part of the Luquillo Experimental Forest, U.S. Forest. The middle third of the watershed is dominated by agricultural land and several housing projects that dump their sewage into the Mameyes River. The lower third of the watershed is dominated by two small towns which contribute municipal, domestic, and light industry wastes to the river. The Mameyes River empties into the Atlantic Ocean near the largest public beach in Puerto Rico, Luquillo. Average daily attendance at Luquillo Beach exceeds 1,000 persons, with holiday crowds sometimes exceeding 10,000. Samples were taken from 6 of 12 sites previously established and characterized in detail by Hazen and Aranda (14). These sites

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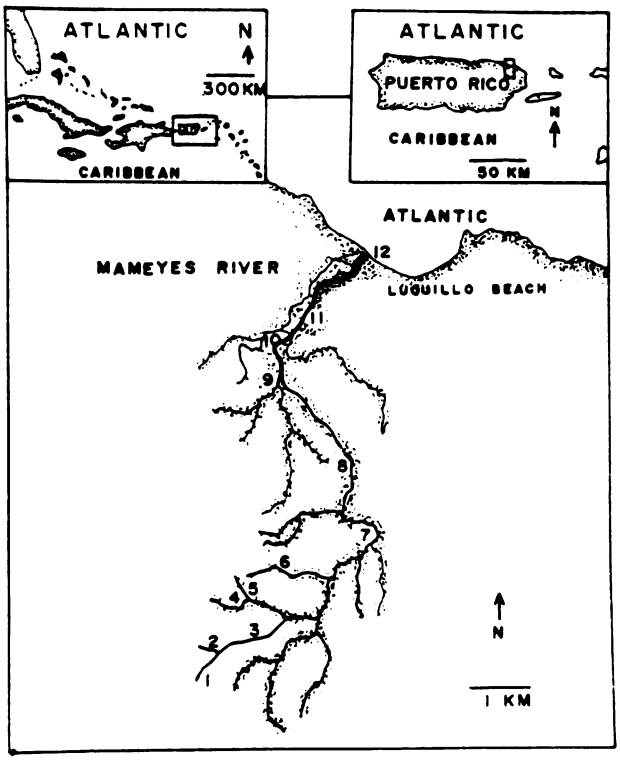

FIG. 1. Map showing location of study sites in Mameyes River watershed, Puerto Rico.

were sampled seven different times during the course of 12 consecutive months, beginning January 1983 with ca. 3 weeks between sampling. Previous studies have shown that this watershed exhibits no seasonal differences in water quality (14).

Water quality. Seven water quality parameters were measured simultaneously with water collection for bacterial density. Dissolved oxygen, pH, air temperature, water temperature, and conductivity were measured in situ with a model 4041 Hydrolab Surveyor (Hydrolab Corp., Austin, Tex.). Measurements for alkalinity and hardness were done at the sampling site with Spectrokits and a mini-spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). Three liters of water were collected and divided into various bottles, and small amounts of the following preservatives were added: sulfuric acid, zinc acetate, and mercuric chloride. Amber bottles were used for samples to be analyzed for chlorophyll. All samples were then placed on ice for transport to the laboratory. The appropriately preserved samples were then analyzed for the following parameters: nitrates plus nitrites, sulfates, phosphates (P_i), total phosphorus, and chlorophyll *a* (using standard methods [2]; for details, see M. Carrillo, M.S. thesis, University of Puerto Rico, Rio Piedras, 1983).

Bacteriological methods. Water samples for *Bifidobacterium* spp. analysis were collected by grab sampling slightly below the surface with sterile, 180-ml Whirl-Pak bags (NASCO, Ft. Wilkinson, Wis.). Samples were transported to the laboratory at ambient temperature; we found this to give better results than transport on ice (M. Carrillo, unpublished data). The time from collection to analysis in the laboratory never exceeded 5 h. Sample portions of 1, 10, 20, 50, and 100 ml were filtered in triplicate with 0.45-μm (pore size), 47-mm-diameter type GN-6 membrane filters (Gelman Instrument Co., Ann Arbor, Mich.). The 1-ml sample was diluted with filter-sterilized phosphate-buffered saline (pH 7) to give better spread on the filter. Each filter was then placed on YN-6 medium (28) in 47-mm, tight-fitting petri plates. YN-6 is a differential, selective medium for *Bifidobacterium* spp. All media preparation, incubation, and enumeration was as described by Resnick and Levin (28). A hot pin was used to put several holes in the cover of each petri dish to allow gas exchange. The plates were incubated at 37°C for 48 h under anaerobic conditions in BBL GasPak jars (BBL Microbiology Systems, Cockeysville, Md.). After incubation, colonies that were 1 to 2 mm in diameter, green, circular, smooth, and butyrous were considered presumptive *Bifidobacterium* spp. Confirmation was based on gram-positive, bifid rod-shaped morphology, i.e., colonies were noncatalase producing, nonnitrate reducing, nonmotile, and able to ferment lactose without gas production. Species identification was confirmed by measuring amounts of acetic and lactic acid produced as by-products of glucose fermentation by gas chromatography (17). A model 3920 TCD gas chromatograph (The Perkin-Elmer Corp., Norwalk, Conn.) fitted with a Chromosorb 1000 stainless steel column (Supelco, Belafonte, Pa.) was used for determining carbohydrate fermentation patterns. The following reference cultures, obtained directly from American Type Culture Collection, were used as controls: *Bifidobacterium adolescentis* ATCC 15703 and *Bifidobacterium longum* ATCC 15707.

Fecal coliform and *E. coli* counts were made from grab samples collected in 180-ml Whirl-Pak bags and transported to the laboratory on ice. Samples of 1, 10, and 100 ml were filtered, with the 1-ml sample diluted with phosphate-buffered saline as before. Filtration was with 0.7-μm (pore size), 47-mm-diameter type HC membrane filters (Millipore Corp., Bedford, Mass.). The filters were placed on mTEC medium (7) and incubated for 2 h at 35°C. After this period of resuscitation, the plates were incubated at 44.5°C for 20 h. After incubation, yellow, yellow-green, and yellow-brown colonies were recorded as fecal coliforms (26). The countable filters were transferred to petri dishes with pads saturated with urea to test for urease production (7). After 15 min of incubation, all yellow colonies were counted as presumptive *E. coli*. Confirmation was with API 20E strips (Analytab Products, Plainview, N.Y.) and citrate utilization. A culture of *E. coli* ATCC 11775 was used as a control for all media and tests.

Total anaerobe counts were made by collecting subsurface water in 180-ml Whirl-Pak bags and transporting them at ambient temperature to the laboratory for analysis. Samples were diluted to 10 ml with anaerobic salt solution as described previously (6) to give effective concentrations of 10^{-5}, 10^{-3}, 10^{-2}, 10^{-1}, and 1 ml. Diluted samples were filtered through 0.45-μm (pore size), 47-mm-diameter type HA membrane filters (Millipore), placed on supplemented anaerobic brain heart infusion agar (BBL), and incubated at 25°C for 24 h, followed by incubation at 35°C for another 24 h. All incubations were under anaerobic conditions with BBL GasPaks, as described by Daily et al. (6).

Cell activity, respiration, and total direct counts were determined by acridine orange direct counting (AODC) and the respiring count technique of Zimmerman et al. (34). Ten milliliters of water sample from each site was incubated with 1 ml of 0.2% 2-(*p*-iodophenyl)-3(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) for 30 min in the dark. The reaction was stopped by fixing with 0.1 ml of 37% formaldehyde. In the laboratory, samples were stained with acridine orange and filtered through 0.22-μm (pore size), 47-mm-

diameter, sudan black B-stained polycarbonate filters (Nuclepore Corp., Pleasanton, Calif.). Filters were examined under oil immersion with a model 2071 epifluorescence microscope fitted with transmitted bright-field illumination (American Optical Corp., Buffalo, N.Y.). The total number of bacteria was determined by counting all fluorescing cells. The percentage of active cells was determined by dividing the number of red fluorescing cells by the total number of fluorescing cells. The percentage of cells that were respiring was determined by dividing fluorescing cells that also had intracellular INT-formazan granules (when viewed by bright-field microscopy) by the total number of fluorescing cells.

Diffusion chamber studies. Pure cultures of *B. adolescentis* (isolated from a sewage outfall on the Mameyes River near site 9) were grown in peptone-yeast-glucose broth (Difco Laboratories, Detroit, Mich.) at 37°C anaerobically for 48 h. Pure cultures of *E. coli* ATCC 11775 were grown in nutrient broth at 37°C for 24 h. Cells were harvested by centrifugation and washed in sterile phosphate-buffered saline. The number of cells per milliliter was determined with a model ZF Coulter Counter (Coulter Electronics, Hialeah, Fla.) and adjusted to 10^8 cells ml^{-1}.

The final bacteria suspension was placed into a sterile diffusion chamber just before immersion at the study site. The chamber had a capacity of 100 ml and a total diffusion surface area of 16,515 mm^2, both of which are modifications (3, 15) of the MSU-DME chamber (22). The diffusion surface for this study was created by a 0.2-mm (pore size), 142-mm-diameter, nylon-reinforced Versapor membrane filter (Gelman Instrument Co.).

Four chambers for each bacteria studied were suspended 0.5 m below the surface at sites 1 and 4 (Fig. 1). Samples (1 ml) were taken with a sterile syringe from each chamber at regular intervals for 72 h. Half of each sample was immediately fixed in 10% phosphate-buffered Formalin (pH 7) and refrigerated for later reading with the Coulter Counter, as described by Hazen and Esch (15). The other 0.5 ml was incubated with INT in the dark for 30 min at the in situ temperature and then fixed with formaldehyde as described above. Later, these samples were enumerated for percent activity, percent respiration, and AODC as described above.

Data analysis. Programs developed on Apple II Plus and IBM 370-148 computers were used for all statistical tests. Two-factor analysis of variance was used to test differences between sites and times. Multiple correlation and regression were used to determine relationships between densities of bacteria and water quality parameters. Heteroscedastic data as determined by skew and kurtosis were made more homoscedastic by transformation with log (x + 1). Any statistical probability greater than or equal to 0.05 was considered significant (33).

RESULTS

Water quality. As reported in other studies (14; A. J. Lopez-Torres, M.S. thesis, University of Puerto Rico, Rio Piedras, 1982; N. Perez-Rosas, M.S. thesis, University of Puerto Rico, Rio Piedras, 1983), water quality in the Mameyes River watershed does not vary seasonally but rather as a function of catastrophic rainfalls which occur irregularly all year. Thus, variability in most of the parameters (e.g., water temperature) is less than 5% (Table 1). In general, there was a gradient in parameter concentrations from the upper parts of the watershed to the lower parts. Water temperature, air temperature, and conductivity were lowest at site 1 and increased up to site 9. Sites 4, 5, and 9

TABLE 1. Water quality in the Río Mameyes watershed[a]

Site no.	Chemical characteristic (mean ± 1 SE)														
	WTEMP	ATEMP	COND	DO	pH	ALK	HARD	CHLA	NO_{2+3}	SO_4	PO_4	TP	TDC	%RC	%AC
1	21.1 ± 0.5	23.1 ± 1.0	73 ± 4.8	7.9 ± 0.2	6.2 ± 0.1	24 ± 4	33 ± 7	148 ± 71	0.43 ± 0.13	5.3 ± 0.7	2.40 ± 0.91	3.48 ± 1.28	18 ± 7	50 ± 12	37 ± 5
3	21.5 ± 0.5	23.4 ± 1.0	79 ± 6.5	7.9 ± 0.2	6.4 ± 0.2	24 ± 4	40 ± 12	37 ± 15	0.40 ± 0.16	3.8 ± 0.8	1.52 ± 0.76	5.20 ± 2.10	29 ± 9	35 ± 7	32 ± 10
4	21.6 ± 0.9	23.6 ± 0.9	114 ± 5.2	8.1 ± 0.2	6.8 ± 0.1	32 ± 5	53 ± 18	47 ± 19	0.67 ± 0.18	8.8 ± 3.1	0.99 ± 0.68	5.69 ± 2.85	38 ± 22	42 ± 12	33 ± 10
5	21.7 ± 0.4	23.7 ± 0.9	156 ± 5.2	8.2 ± 0.2	7.2 ± 0.1	48 ± 5	73 ± 24	77 ± 29	0.53 ± 0.13	15.3 ± 4.3	1.36 ± 0.95	3.04 ± 1.17	20 ± 4	40 ± 9	34 ± 8
7	24.2 ± 0.6	27.2 ± 2.2	113 ± 7.7	8.2 ± 0.3	7.0 ± 0.1	36 ± 4	67 ± 24	41 ± 4	0.50 ± 0.12	10.5 ± 3.2	0.75 ± 0.29	1.82 ± 0.21	61 ± 39	37 ± 10	43 ± 12
9	27.1 ± 0.9	28.2 ± 1.4	194 ± 6.0	6.9 ± 0.5	7.0 ± 0.1	68 ± 14	187 ± 64	106 ± 63	1.40 ± 0.71	10.6 ± 1.8	6.83 ± 2.34	8.96 ± 2.61	65 ± 39	43 ± 14	38 ± 18

[a] All values are the result of seven samples. Abbreviations: WTEMP, water temperature (degree Celsius); ATEMP, air temperature (degree Celsius); COND, conductivity (S per centimeter); DO, dissolved oxygen (milligrams per milliliter); ALK, alkalinity (milligrams of $CaCO_3$ per milliliter); HARD, hardness (milligrams of $CaCO_3$ per milliliter); CHLA, chlorophyll *a* (milligrams per milliliter); NO_{2+3}, nitrites plus nitrates (milligrams per liter); SO_4, sulfates (milligrams per liter); PO_4, phosphates (P_i [micrograms per liter]); TP, total phosphorus (micrograms per liter); TDC, total direct count by AODC (CFU per milliliter × 10^4); %RC, percentage of respiring cells by INT; and % AC, percentage of active cells by AODC.

TABLE 2. Densities of bacteria and their ratios by site[a]

Site no.	Density (mean ± 1 SE) of:						
	FC	EC	B	TAC	B/F	B/E	B/TA
1	1.3 ± 0.8	1.0 ± 0.7	5.6 ± 1.9	453.0 ± 44.9	11.3 ± 4.3	16.6 ± 7.5	24.4 ± 14.3
3	0.7 ± 0.2	0.3 ± 0.1	3.4 ± 1.4	9.5 ± 7.1	4.2 ± 1.6	22.7 ± 11.2	15.6 ± 13.9
4	0.2 ± 0.2	0.1 ± 0.0	0.3 ± 0.1	1.2 ± 0.5	1.8 ± 0.9	3.9 ± 1.9	6.2 ± 2.8
5	0.1 ± 0.0	0.1 ± 0.0	0.6 ± 0.3	3.6 ± 2.0	3.5 ± 1.5	4.0 ± 1.4	3.7 ± 2.5
7	1.9 ± 1.2	0.4 ± 0.2	1.3 ± 0.9	2.0 ± 0.6	2.1 ± 1.1	17.1 ± 15.3	12.0 ± 5.7
9	184.0 ± 87.0	134.0 ± 66.0	6.5 ± 1.6	126.0 ± 64.0	0.2 ± 0.1	0.4 ± 0.2	5.9 ± 3.5

[a] Abbreviations: FC, fecal coliform; EC, *E. coli*; B, bifidobacteria; TAC, total anaerobes × 10^2; B/F, bifidobacteria/fecal coliform; B/E, bifidobacteria/*E. coli*; and B/TA, bifidobacteria/total anaerobes × 10^{-3}. All units are in CFU per milliliter.

had high values for alkalinity and hardness. Site 5 had the highest pH, whereas sites 1 and 3 had the lowest pH. Site 9 had the lowest concentration of dissolved oxygen and chlorophyll *a* but was high in phosphates, total phosphorus, nitrates plus nitrites, and sulfates. Site 9 receives effluent directly from a primary sewage treatment plant and thus was the most polluted, as indicated by water quality (Table 1). Site 1 had higher nitrates plus nitrites, phosphates, sulfates, and chlorophyll *a* concentrations than the sites immediately below it in the watershed. Other studies by our laboratory (C. F. Aranda, M.S. thesis, University of Puerto Rico, Rio Piedras, 1982) have shown that incident radiation is higher at site 1 due to a less well-developed forest canopy.

Bacteria distribution and abundance. The highest densities of fecal coliforms, *E. coli*, and *Bifidobacterium* spp. were found at site 9, the sewage point source (Table 2). However, site 1 had significantly higher densities for each of these bacteria than the sites below it in the watershed, except for site 9. In addition, site 1 had the highest density of total anaerobes of any of the sites. The ratio of bifidobacteria to *E. coli* and the ratio of bifidobacteria to fecal coliform bacteria was significantly lower at site 9 than any of the other sites. The ratios of bifidobacteria to total anaerobic bacteria were not significantly different between sites. Direct counts of bacteria increased through the watershed, with site 1 having the lowest AODC and site 9 the highest. However, percent bacterial respiration as measured by INT was greatest at site 1 (Table 1). Percent activity of the bacterial community was not significantly different between sites.

A total of 116 presumptive *Bifidobacterium* spp. colonies were isolated and analyzed; 105 (90.5%) were confirmed by morphological and biochemical tests. Gas chromatography was used to analyze 22 presumptive and biochemically confirmed isolates; 86.2% of these were confirmed by the gas chromatograph method. Thus, the YN-6 medium gave 21.8% false-positives. All 20 presumptive negative colonies tested were confirmed as negative; thus, YN-6 showed 0% false-negatives. The Mameyes River watershed showed the following bifidobacteria species from the 105 isolates: *B. adolescentis* (88.9%), *B. angulatum* (5%), *B. infantis* (5%), and *Bifidobacterium* spp. (unknown) (1.1%).

Bacterial survival in situ. The density of *E. coli* as measured by the Coulter Counter increased significantly over time ($F = 16.5$, df = 10 and 21, $P < 0.0001$) but was not significantly different between sites (Fig. 2). Densities of *B. adolescentis*, on the other hand, decreased significantly over time ($F = 47.5$, df = 10 and 21, $P < 0.0001$), and there were no significant differences between sites (Fig. 2). Densities as measured by AODC for *E. coli* were significantly different by site ($F = 9.83$, df = 1 and 21, $P < 0.01$), with site 4 having a

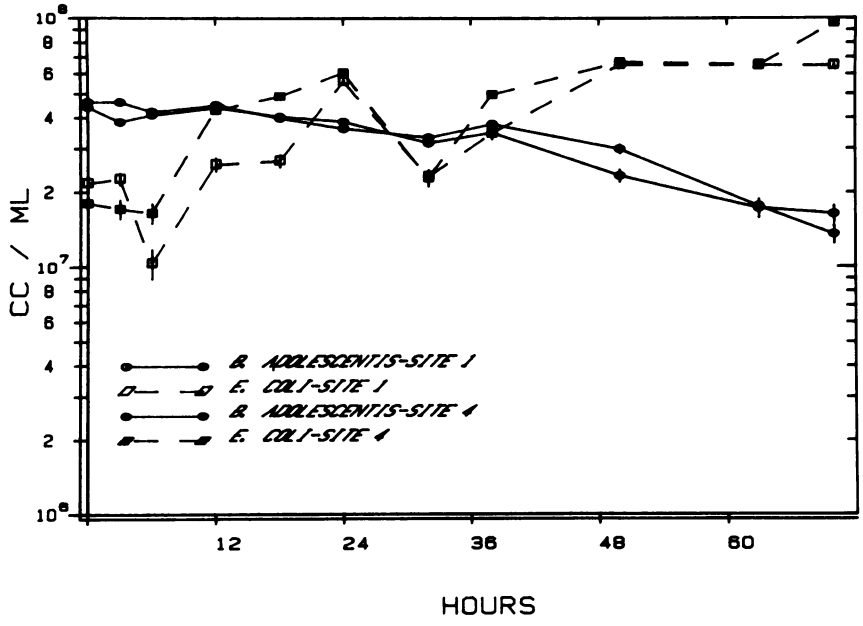

FIG. 2. Survival in situ of *B. adolescentis* and *E. coli* at site 1 and site 4 (mean density by Coulter Counter ± 1 standard error, $n = 4$).

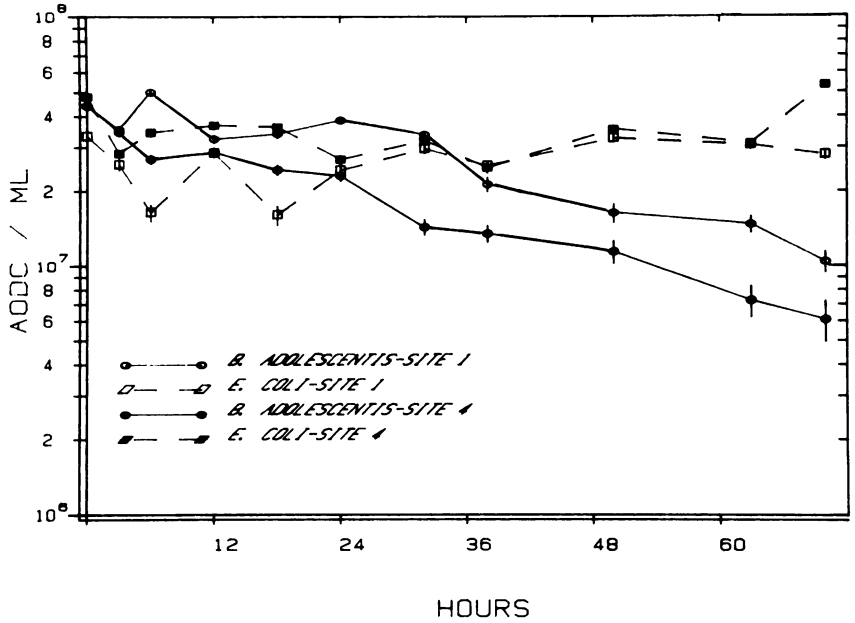

FIG. 3. Survival in situ of *B. adolescentis* and *E. coli* at site 1 and site 4 (mean density by AODC ± 1 standard error, $n = 4$).

slightly greater regrowth than site 1 (Fig. 3). Differences over time were not significant for densities of *E. coli* as measured by AODC. Densities of *B. adolescentis* were also significantly different by site ($F = 25.3$, df = 1 and 21, $P < 0.001$) when measured by AODC (Fig. 3). Site 4 showed a much more rapid decrease in density of *B. adolescentis* over time than did site 1. Densities of *B. adolescentis* as measured by AODC also decreased significantly over time ($F = 16.9$, df = 10 and 21, $P < 0.001$) at both sites.

The percent activity of the *E. coli* population in the chambers as measured by AODC was significantly greater at site 1 ($F = 10.6$, df = 1 and 21, $P < 0.01$); however, there were no significant differences over time due to the large variability (Fig. 4). The percent activity of the *B. adolescentis* population was significantly greater at site 4 ($F = 8.0$, df = 1 and 21, $P < 0.02$), with no significant differences occurring over time (Fig. 4). The percentage of the *E. coli* population that was respiring did not change significantly over time or by site. However, the proportion of the *E. coli* population that was respiring declined from 60 to <30% within the first 3 h in the chambers at both sites. Little change occurred after this, i.e., less than 10% over the remaining 69 h of the study. The *B. adolescentis* population did not show any significant respiration at either site during

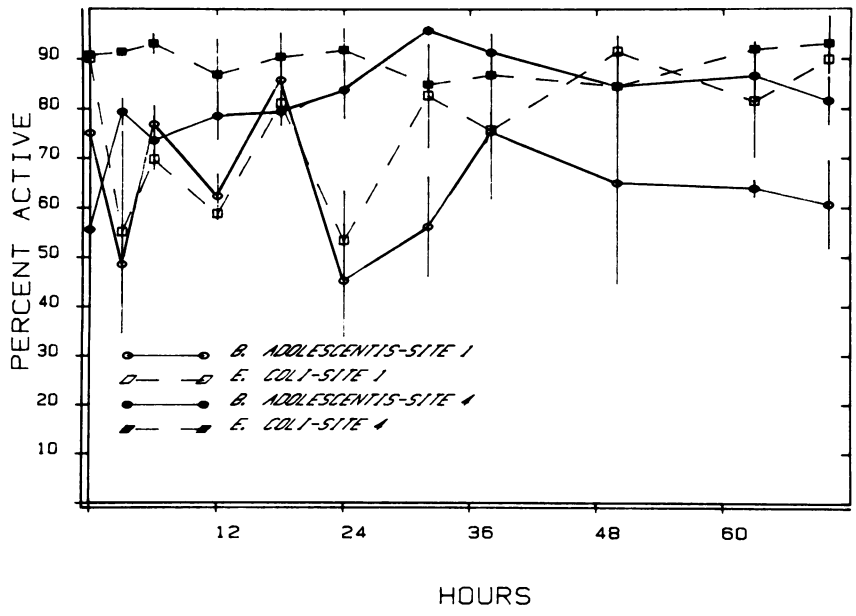

FIG. 4. Activity change in situ of *B. adolescentis* and *E. coli* at site 1 and site 4 (mean percent AODC activity ± 1 standard error, $n = 4$).

TABLE 3. Correlation half-matrix of water quality[a]

Chemical characteristic	WTEMP	ATEMP	COND	DO	pH	ALK	HARD	CHLA	NO_{2+3}	SO_4	PO_4	TP	FC	EC	B	TDC	%RC	%AC	TAC
WTEMP	1.000																		
ATEMP	0.714	1.000																	
COND	0.519	0.149	1.000																
DO	−0.745	−0.423	−0.291	1.000															
PH	0.400	0.443	0.461	−0.181	1.000														
ALK	0.127	−0.021	0.030	−0.016	−0.085	1.000													
HARD	0.622	0.495	0.063	−0.607	0.335	0.469	1.000												
CHLA	0.033	0.209	−0.144	−0.047	0.294	−0.050	0.247	1.000											
NO_{2+3}	0.374	0.356	0.082	−0.305	0.492	−0.168	0.521	0.467	1.000										
SO_4	0.318	0.506	0.206	−0.111	0.413	−0.057	−0.264	0.053	0.143	1.000									
PO_4	0.437	0.276	0.109	−0.513	0.004	0.255	0.510	0.005	0.318	0.015	1.000								
TP	0.341	0.344	0.084	−0.386	0.135	0.068	0.389	0.002	0.418	0.128	0.707	1.000							
FC	0.634	0.441	0.301	−0.460	0.140	0.419	0.628	0.026	0.387	0.070	0.630	0.451	1.000						
EC	0.615	0.440	0.294	−0.490	0.152	0.450	0.645	0.100	0.389	0.104	0.668	0.489	0.976	1.000					
B	0.291	0.027	0.061	−0.381	−0.345	0.265	0.324	−0.064	0.088	−0.095	0.469	0.190	0.501	0.480	1.000				
TDC	0.047	0.054	−0.021	0.171	−0.082	0.315	0.101	−0.152	−0.033	−0.075	0.206	0.193	0.361	0.283	−0.053	1.000			
%RC	0.056	−0.163	0.168	−0.185	−0.042	0.007	−0.025	−0.161	0.003	−0.346	−0.103	−0.037	−0.088	−0.115	−0.058	0.172	1.000		
%AC	0.282	0.245	0.106	−0.208	0.121	0.138	0.231	0.196	0.265	0.181	−0.002	−0.148	−0.082	−0.061	0.159	0.500	0.462	1.000	
TAC	0.412	0.351	0.085	−0.497	0.017	0.035	0.521	0.271	0.403	0.059	0.651	0.463	0.504	0.545	0.456	0.003	0.047	0.233	1.000

[a] $P < 0.05$ and $r > 0.301$. See Tables 1 and 2 for abbreviations. Underlined values are significant.

the entire study period, i.e., less than 4% at either site at any time measured.

Water quality and bacterial abundance. A multiple correlation analysis revealed that *Bifidobacterium* spp. were positively correlated with hardness, phosphates, and densities of fecal coliforms, *E. coli*, and total anaerobes (Table 3). These same bacteria were negatively correlated with dissolved oxygen and pH. Fecal coliforms were correlated to the same parameters in the same way but were also positively correlated with water and air temperature, conductivity, alkalinity, hardness, nitrates plus nitrites, phosphates, and total phosphorus. Densities of *E. coli* and total anaerobes showed the same correlations with water quality as did fecal coliforms. AODC was positively correlated with alkalinity and density of fecal coliforms. The percentage of the bacterial community that was respiring was negatively correlated with sulfate concentration and positively correlated with the percentage of the bacterial community that was active, as measured by AODC.

DISCUSSION

The Mameyes River watershed is relatively oligotrophic despite the fact that the source of the watershed is a cloud tropical rain forest. (For a complete description of all sites and a thorough discussion of trophic status of this watershed see Hazen and Aranda [14] and C. F. Aranda, M.S. thesis, University of Puerto Rico, Rio Piedras, 1982.) Concentrations of total phosphorus, alkalinity, phosphates, and nitrates plus nitrites are within the range of values for oligotrophic-mesotrophic freshwaters (32). Site 9 and site 1 were the only sites that were not low in nutrients, e.g., phosphates, total phosphorus, and nitrates plus nitrites. Site 9 was the point source for a primary sewage treatment plant effluent; thus, the eutrophic nature of this site is obvious. Site 1, on the other hand, is the highest point sampled in the watershed. The increased concentrations of some nutrients at this site may be explained by the higher algal densities observed. Higher algal densities at this site could be the result of a thinner forest canopy in the upper part of the rain forest which allows sunlight to reach the river. Further down in the watershed, the forest canopy is dense enough to inhibit algal growth (C. F. Aranda, M.S. thesis, University of Puerto Rico, Rio Piedras, 1982). Another possibility is that slightly more nutrients are leached into the river at the top of the watershed because of higher levels of biodegradation of leaf litter and lower levels of nutrient adsorption by the forest standing crop (25). This is indicated by the dwarf nature of the forest. Trees that normally reach >20 m are 5 m or less in the dwarf forest at the top of the watershed. Wind action appears to be primarily responsible for this dwarfing, which also increases leaf fall rate (M. Byer, University of Puerto Rico, personal communication).

Torrential rainfalls (>10 cm h^{-1}), which can cause the river level to change more than 2 m in less than an hour, are the principal cause of the low densities of resident flora and fauna and the lack of seasonality in all parameters (C. F. Aranda, M.S. thesis, University of Puerto Rico, Rio Piedras, 1982). Temperature is quite constant throughout the year, and rainfall does not exhibit any consistent pattern in the area.

Bacteria distribution and abundance. The highest densities of fecal coliforms, *E. coli*, and *Bifidobacterium* spp. were recorded at site 9, the sewage outfall. In addition, the lowest ratios between bifidobacteria and fecal coliforms and between bifidobacteria and *E. coli* were at site 9. Densities of

fecal coliforms reported in this study were lower than those reported by Evison and James (8) for river samples taken in two countries in tropical Africa and much higher than those reported for river samples taken in England. In general, the site which received sewage effluent had densities of fecal coliforms and *E. coli* that were 2 orders of magnitude higher than uncontaminated sites. However, the densities of fecal coliforms and *E. coli* at all sites exceeded recommended coliform MCL for potable waters, i.e., 0.04 CFU ml^{-1} (10). Site 9 also exceeded the recommended fecal coliform MCL for primary contact recreational waters, i.e., 2 CFU ml^{-1} (5). Note that while the MCL is for total coliforms, our measurements were of fecal coliforms and *E. coli*. Both are considered subsets of total coliforms. Indeed, in this study and in previous studies, we have found that analyses for total coliforms are consistently overgrown at the dilutions used in this study. Lavoie (20) found that total and fecal coliform assays did not have good resolution in tropical climates. Thus, standard indicators such as fecal coliforms and *E. coli* function only in terms of relative numbers in this tropical system, not in terms of absolute densities when compared with temperate systems.

Bifidobacteria as enumerated with the YN-6 medium gave high background counts and did not show large increases at sites known to contain sewage contamination. Gyllenberg et al. (11) and Evison and James (8) showed that densities of bifidobacteria were always greater than densities of *E. coli* or fecal coliforms in contaminated waters. In the rain forest, all sites had higher densities of bifidobacteria than *E. coli* and fecal coliforms, except site 9, the sewage outfall. Studies of isolates suggested that much of the difficulty may be inherent in the YN-6 medium itself, since 21.8% false-positives were found. Resnick and Levin (29) obtained 100% confirmation of 53 environmental isolates obtained in temperate areas. The bifidobacteria/*E. coli* ratio obtained by Resnick and Levin (29) at sewage outfalls was between 2.3 and 14.4, and the ratio was always less than 1.0 for nonpoint source waters. Our study showed quite the opposite. Only the point source had bifidobacteria/*E. coli* ratios of less than 1, whereas all of the nonpoint source samples had bifidobacteria/*E. coli* ratios that were greater than 1.0. The same pattern was seen when a less specific indicator like fecal coliform density was compared with bifidobacteria densities.

Total anaerobic counts were highest at site 1. This also suggests increased biodegradation and leaching from the dwarf forest at the top of the watershed. Since the ground is saturated with water and therefore anoxic, the dominant soil bacterial component is anaerobic. Higher rates of bacterial community respiration and activity at site 1 also suggest a recent source of nutrients, especially since the total bacterial density (AODC) is significantly lower at site 1 than the sites farther down in the watershed. In comparison to temperate total anaerobic counts, Daily et al. (6), using the same techniques, found 2×10^2 to 9×10^3 CFU ml^{-1} in the Anacostia River, Washington, D.C. This grossly polluted, cold, temperate river had lower densities of total anaerobes than most of the rain forest sites. Indeed, sites 1 and 9 had higher densities of total anaerobic counts than any reported by Daily et al. (6).

Survival of bifidobacteria and water quality. Both direct counting methods of the in situ diffusion chamber densities demonstrated that *E. coli* could survive indefinitely in the rain forest watershed. Regrowth was evident by both methods. Other studies have suggested that *E. coli* may be able to survive and regrow for extended periods in tropical habitats (4, 8). In temperate environments, it is well documented that *E. coli* does not survive well in freshwater (21, 22, 30). This characteristic of nonsurvival in extraenteral environments is one of the major underlying assumptions of the use of coliforms as indicators of fecal contamination (4). Thus, *E. coli* and therefore coliforms would seem to be invalid as indicators of recent fecal contamination in tropical waters. In contrast, densities of *B. adolescentis* decreased significantly over time in the chambers as measured by either direct count method. Densities of *B. adolescentis* declined more than 50% in 48 h. Resnick and Levin (29) showed that bifidobacteria in the laboratory (held in filter-sterilized freshwater at 4, 12, and 20°C) declined more than 80% in cell density in only 24 h. The in situ conditions of the tropical rain forest river seem much more conducive to prolonging bifidobacteria survival.

Site 1, which had higher nutrient levels (i.e., total phosphorus, phosphates, and nitrates plus nitrites), supported lower densities of *E. coli* but had higher rates of survival of *B. adolescentis*. Since nutrient levels and algal density were higher at site 1, the best possible explanation is that microbial antagonism was a major factor. It has been established that bifidobacteria and other anaerobes can inhibit *E. coli* (23, 27). Site 1 also had the highest densities of total anaerobes. This, coupled with a slight nutrient advantage for *B. adolescentis* at site 1, would explain the between-site differences observed in bacterial survival.

The diffusion chamber activity and respiration measurements showed that *E. coli* is active (>50% at any time) and respiring (>10% at any time). The percentage of the population that was respiring was quite high, i.e., 5 to 36%, compared with environmental samples taken by others (33). The respiration and activity of both bacteria in the diffusion chambers was also greater than the ambient bacterial community rates. This would indicate that either acclimation was incomplete for the chamber populations or that large numbers of other bacteria with much lower rates of respiration and activity were present in the ambient community. The percentage of the *E. coli* population that was active was greatest at site 4, even though this site supported lower densities of *E. coli* as measured by direct count. This further suggests microbial antagonism. The activity of *B. adolescentis* was also highest at site 4. The large variability between sampling times for site 1 for both bacteria indicates that this site is quite unstable compared with site 4. At site 4, the bacteria seem to physiologically stabilize after only 24 h. Other studies by our laboratory have shown that most bacteria measured by these techniques physiologically stabilize quite quickly in unpolluted tropical waters but have much greater difficulties in grossly contaminated waters (N. Perez-Rosas, M.S. thesis, University of Puerto Rico, Rio Piedras, 1984; A. J. Lopez-Torres, M.S. thesis, University of Puerto Rico, Rio Piedras, 1982; I. Lopez de Cardona, M.S. thesis, University of Puerto Rico, Rio Piedras, 1984).

Analysis of water quality and viable counts revealed correlations between viable counts of fecal coliforms and temperature, alkalinity, nitrates plus nitrites, and total phosphorus. Since these parameters are indirectly or directly sources of nutrients for these bacteria the relationship would seem to be one of cause and effect. Numerous other models have shown similar relationships (12, 13, 15, 16). However, it is possible that sewage effluents, which are high in these same parameters and high in coliforms of fecal origin, could cause the observed correlations in the absence of a true cause-effect correlation. In this study, only site 9 had sewage input, and if this site were eliminated from the data matrix,

the correlation matrix would not be significantly changed. Indeed, higher nutrients at site 1 resulted in higher densities of all viable counts in the absence of sewage. Thus, the primary regulator of densities of indicator bacteria in this tropical environment is probably the nutrient concentration of the water. The correlation between bifidobacteria and water hardness is explained by the work of Kojima et al. (19), who found that calcium ions can be a significant growth factor for bifidobacteria in culture.

This study suggests that coliforms may not only survive but become normal flora in tropical freshwater environments. Certainly coliforms would seem to be poor indicators of recent human fecal contamination, according to the criteria of Bonde (4). These findings raise serious doubts as to the efficacy of coliform MCL as the sole criteria for determining the usability of tropical freshwaters. Bifidobacteria show promise as an indicator of recent fecal contamination in terms of lack of survival in situ and specificity as a human fecal indicator. Unfortunately, the currently available medium for enumeration (YN-6) is hampered by a lack of specificity and insufficient resolution when background bacterial densities are high. Considering the importance of fecal contamination of freshwater in the dissemination of diseases to humans in tropical areas, we must continue to search for better methods and indicators that work over a range of different tropical environments.

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